BACILLUS CEREUS PHAGE ISOLATED FROM RAW MILK

IDENTIFICATION:

A RESEARCH PROJECT

SUBMITTED TO THE GRADUATE SCHOOL

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE

MASTER OF ARTS

BY

KYLA L.ADAMSON

DR. JOHN MCKILLIP - ADVISOR

BALL STATE UNIVERSITY

MUNCIE, INDIANA

DECEMBER 2018

INTRODUCTION:

Bacillus cereus

Bacillus cereus (*B. cereus*) is a Gram-positive, rod- shaped, aerobic- to facultatively anaerobic bacterial species [1] that belongs to the *Bacillus cereus* group, which also consists of *B. anthracis*, *B.thuringiensis,* and *B. weihenstephanensis [2]*. These three bacterial species are very similar phenotypically. In fact, *B.thuringiensis* can only be differentiated from *B. cereus* by the presence of genes coding for insecticidal proteins (Cry toxins) in the former. Cry toxins are crystalline structured proteins that have been used in commercial insecticides since the mid 1900s [3]. *B. cereus* had long been regarded as simply a contaminant in clinical samples, and a spoilage organism in food products[1]. However, in the last 50 years*, B. cereus* has been found to cause a plethora of human infections. Presently, *B. cereus* is most commonly associated with gastrointestinal illnesses such as emesis and diarrheal syndrome. This type of intoxication or toxico-infection generally occurs through the ingestion of viable endospores or vegetative cells from contaminated food at densities at or above ~10^5/g.

The high-throughput nature of the dairy industry necessitates sensitive and selective assays to rapidly detect pathogens. According to the CDC, an estimated 48 million cases of foodborne illnesses occur each year. It is unclear how many cases occur worldwide due to the fractured nature of the data, as well as the prioritizing of particular foodborne organisms [4]. The presence of unwanted organisms in food not only poses a risk to public health, but also costs food industries millions of dollars a year due to food spoilage, or undesired physical changes to a food product that make it no

longer edible. Even with modern preservation practices, approximately 25% of food is lost in the United States due to microbial spoilage.

The ubiquitous nature of *Bacillus cereus* combined with its ability to produce endospores that are highly adhesive makes these bacteria an ever present problem in food production, packaging, and shipment/storage [5]. Furthermore, although the optimum temperature for a vegetative *B. cereus* cell is between 35 and 40^{o}C, the endospores formed by *B. cereus* are resistant to high temperatures and desiccation, allowing them to survive many quality control practices such as pasteurization and dehydration. Subsequently, even if pasteurization effectively kills all viable cells, when the process is complete the undamaged endospores germinate into viable cells that are able to cause disease.

B. cereus relies heavily on exoenzymes as well as enterotoxins to cause tissue destruction [1]. *B. cereus* has been found to produce three pore-forming enterotoxins, and one heat stable emetic toxin. Hemolysin BL (HBL), nonhemolytic enterotoxin (NHE), and cytotoxin K are secreted when vegetative cells, or endospores are ingested. Once released, these enterotoxins induce diarrheal syndrome. Conversely, the emetic toxin, termed cereulide, can be formed in food products, and excreted, thus *B. cereus* can still cause emesis even if no viable cells are ingested. *B. cereus* has also been found to cause infections of the lungs eye, skin, liver, heart, central nervous system and urinary tract using many of the same toxins.

Bacteriophages

Bacteriophages (phages) are viruses that have bacterial hosts. Phages are numerous and make up the largest group of viruses, having been detected in a myriad of natural environments [6]. Phages exhibit a wide range of morphologies, including, tailed, filamentous, cubic, and pleomorphic Phages are also characterized based on genomics (Table 1). Phage genomes may be either RNA or DNA, and either can be single stranded or double stranded. RNA phages can be further characterized by whether or not their genomes are segmented, or packaged in fragments within the capsid [7].

Table 1. Overview of bacteriophage families

Shape	Order or family	Nucleic acid, particulars, size	Member
	Caudovirales	dsDNA (L), no envelope	
	*Myoviridae*	Tail contractile	T4
	*Siphoviridae*	Tail long, noncontractile	λ
	*Podoviridae*	Tail short	T7
	*Microviridae*	ssDNA (C), 27 nm, 12 knoblike capsomers	ϕX174
	*Corticoviridae*	dsDNA (C), complex capsid, lipids, 63 nm	PM2
	*Tectiviridae*	dsDNA (L), inner lipid vesicle, pseudo-tail, 60 nm	PRD1
	*Leviviridae*	ssRNA (L), 23 nm, like poliovirus	MS2
	*Cystoviridae*	dsRNA (L), segmented, lipidic envelope, 70–80 nm	ϕ6
	*Inoviridae*	ssDNA (C), filaments or rods, 85–1950 x 7 nm	fd
	*Plasmaviridae*	dsDNA (C), lipidic envelope, no capsid, 80 nm	MVL2

From: Ackermann, 2007. C, circular. L, linear

Lytic vs Lysogenic Replication Cycles:

Phages can be further grouped based on the type of replication cycle they undergo [8]. For example, some phage can be virulent while others are termed temperate. If a phage is virulent, it is only able to undergo a lytic replication cycle, wherein the phage would attach to its bacterial host, and inject its genetic material into the bacterium. Once inside, the phage hijacks the cell's machinery to translate phage proteins. Once the proteins are made, they are assembled within the host, and the fully

assembled phages are released through lysis of the host. The process then repeats when the mature phage recognizes another host cell, attaches, and enters. Phages can also be temperate. A temperate phage undergoes lysogenic replication.

Lysogenic replication differs from lytic replication in that the phage can undergo an alternate pathway that does not involve immediate lysis of the host. The phage must still recognize and attach to its host bacterium, as well as inject its genetic material into the host. Once injected, the phage DNA may become incorporated into the bacterial host's genome, generating a prophage. If this happens, the bacterium can replicate normally many times until a specific environmental stressor occurs, triggering lysogenic induction, which leads back to the lytic pathway. Lytic phages are often easier to find, as they can be detected through simple plaque formation assays much more quickly than lysogenic phage (Figure 1).

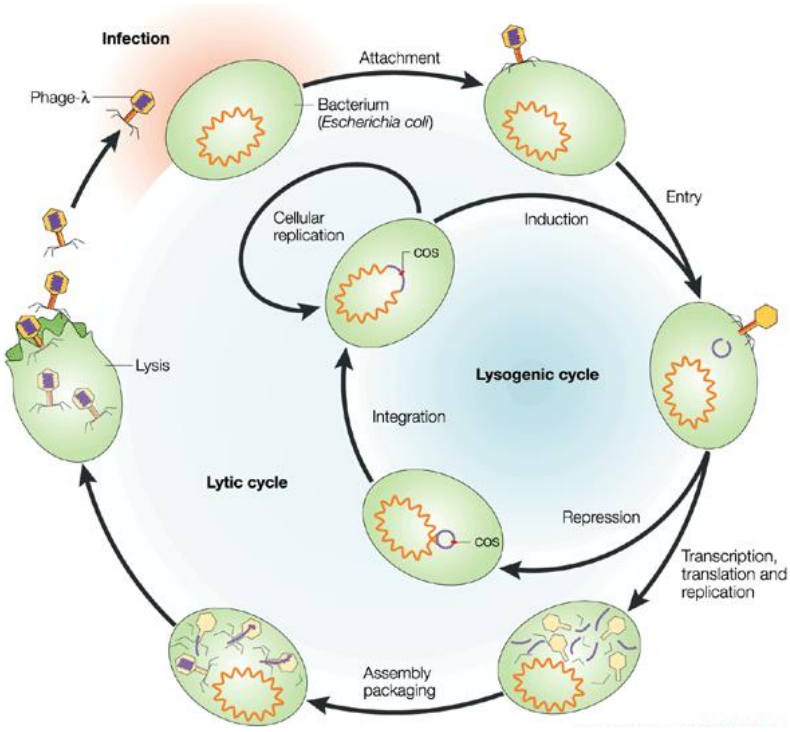

Figure 1: The lytic and lysogenic cycles of a lambda phage. The steps of these cycles are relatively conserved among most types of phages.
(http://www.nature.com/nrg/journal/v4/n6/full/nrg1089.html)

Phage Therapy and Biocontrol:

Phages also have a very specific host range, and are often only able to infect and lyse a single species or subspecies of bacteria [5]. Because of this selective toxicity, they have been considered as a possible way to treat bacterial infections in humans and other animals. This treatment is called phage therapy, and has been investigated essentially since phages were discovered [8]. The selectively toxic nature of many phages means that not only would the patient receiving treatment not be

affected, but nontarget beneficial bacteria would also be unaffected by treatment. The killing of nontarget commensal bacteria has long been a problem with many antibiotic treatments, making phage theoretically a better alternative in this instance. Another characteristic of phages that make phage therapy an attractive means to treat infection is their ability to coevolve with their host, reducing the occurrence of phage-resistant bacteria. Phages can also be used to reduce the number of bacteria in areas outside of medicine, like the food industry. This type of treatment is termed phage biocontrol. In the past, phages as a therapy or as a biocontrol agent were studied, but with the emergence and spread of antibiotic resistance, interest in phages has increased [9].

Phages are most effective when used in cocktails, which is a mixture of different phages that have the same host. Using a cocktail not only further reduces the risk of host resistance, but improves the efficacy of treatment by widening host range, and the mechanism of lysis [10]. The phage based treatment Listshield^{tm} is the first commercially available product to use a phage cocktail to treat listeria in meat .Phage cocktails have also been used as biocontrol methods for other bacteria, such as *E. coli* in meat products [11] *Salmonella* in livestock [12].

Setbacks and Concerns for Phage Therapy:

Some concerns still exist when it comes to implementing phage control. The first is that many factors have influence over the ability of phage to lyse host cells - i.e., temperature, pH, and bacterial density [10]. Even the slightest deviation from the phage's preferred conditions can result in ineffective treatment. Whether the phages are being used to treat humans, animals or animal products, these factors cannot always be

controlled or predicted. Additionally, if the phage is a temperate phage, it is difficult to predict which cycle the phage will undergo, further making therapy less effective and predictable. Another concern is the unwanted transfer of virulence genes to host bacteria [9]. Many bacteria develop virulence factors as a result of the presence of a prophage that has been incorporated into the bacterial genome through lysogeny. It is possible that during the phage replication process, horizontal gene transfer could spread virulence factors. Both of the abovementioned problems can be reduced if not eliminated by carefully selecting the phages in each cocktail and not including any temperate phages.

There are also concerns with phage gene activity occurring within mammalian cells. Mammalian cells have also been found to take up phage DNA and incorporate it into their genomes [9]. When given phages orally, it was found that a small number cells in the digestive tract of mice took up the phage DNA and could even incorporate it into their genomes. However, due to the vastly different cellular mechanisms between a eukaryotic cell and a bacterial cell, there is no indication that the intricate processes required for phage replication could occur in a eukaryotic cell.

Phages as a Detection Assay for Bacteria:

Many of the same factors that make a phage an attractive candidate for the reduction of bacteria in an environment also make them a good candidate for detecting unwanted bacteria. The sensitive and selective nature of phages, as well as their sheer abundance and diversity make them a favorable tool for the detection of almost any kind of bacteria [13]. Generally, most assays used to detect pathogenic

bacteria are lengthy. This is due to the need for an enrichment and/or an amplification step to allow the bacteria to become more numerous and reach detectable levels. Because of the highly efficient nature of the food processing industry, time is valuable when testing for pathogens and food spoilage organisms. The sensitivity of most phages, when applied to a detection assay, could reduce the time needed to detect such bacteria. Phage based detection assays already exist for bacteria such as *E. coli* O157:H7, *Mycobacterium tuberculosis* and *Listeria.* Furthermore, phage based detection assays have been developed to detect spores of the highly infectious *Bacillus anthracis.* We have found no literature, however on a phage-based method to detect *Bacillus cereus* spores in food.

The aim of this study was to screen both milk and soil samples for novel phages that infect *Bacillus cereus,* which could be studied further as prospective candidates to be used in phage therapy or phage detection assays within the dairy industry. We were able to isolate one phage from the milk of a dairy cow that was diagnosed with mastitis caused by *Bacillus cereus.* We then anticipated to be able to sequence parts of its genome and compare it to other known phages on Genbank, and characterize it morphologically using electron microscopy.

MATERIALS AND METHODS:

PHAGE ISOLATION:

Soil Samples:

In order to collect samples to screen for phages, locations around Ball State University were selected. Locations of soil samples were selected with the main priority being diversity in soil type. Samples were collected by using a sterile scoopula to dig approximately 1 inch into the soil. The mass of soil collected varied due to the soil composition. Once collected, samples were immediately added to equal volume of phage buffer (10 mM Tris-HCl, 10 mM $MgSO_4$, 1 mM $CaCl_2$, 6.85 mM NaCl). The samples were then inoculated with early log phase *Bacillus cereus* ATTC14579 (VWR, West Chester, PA) in a liquid culture of 2X Tryptic Soy Broth (TSB) (Weber Scientific, Hamilton, NJ) and allowed to incubate for 12 hours at 23^{o}C. This enrichment step was to increase the likelihood of detecting phages if they were present in low numbers. After enrichment the samples were centrifuged at 4000 x g for 1 minute to sediment the solids. Next, each sample was divided into two aliquots. One of these aliquots was filtered through a 0.2 micron filter, and the other was unfiltered in order to not miss prophages. The samples were added to equal parts of a 12 hour broth culture of *Bacillus cereus* ATTC14579 and incubated for 12 hours at 37^{o}C shaking at 150 rpm.

Milk Samples:

Milk Samples were collected from All Wright Dairy farms in Muncie Indiana. Cows were chosen at random with the exception of one that had been diagnosed with mastitis caused by *Bacillus*. Raw milk was collected directly from the udder of the cow into a sterile container and maintained at 4 deg. C until being processed in the

laboratory. The milk samples were immediately returned to the lab and inoculated with late log phase *Bacillus cereus* ATTC14579 in 2X TSB, and incubated for 12 hours at 37^{o}C shaking at 150 rpm. From this point on, the milk samples were treated the same as the soil samples with the omission of the centrifugation step.

Plating Samples:

One milliliter of each sample was added to 10 mL of molten Tryptic Soy Agar (TSA) (Weber Scientific). Calcium chloride was added to a final concentration of 5mM, as well as 500 mL of 4 hour *Bacillus cereus* in 2X TSB. The molten agar tube was then overlaid on a plate of TSA. Once solidified, the plates were incubated at 23^{o}C and then incubated for 24 hours. After 24 hours the plates were inspected for plaques. Plaques were purified by lifting a single plaque from the agar, adding it to late log phase *Bacillus* in 2X TSB, plating as described above. Plaques were purified 3 times.

DNA EXTRACTION:

Individual plaques were selected, lifted, and added to late log phase *Bacillus cereus* along with a final concentration of 5mM $CaCl_2$, and incubated at 37^{o}C shaking at 150 RPM for 24 hours. Next, the samples were placed in the centrifuge and spun at 14,000 x g for 10 minutes at room temperature. The supernatant was retained in a separate tube. The pellet was resuspended in water, and treated with lysozyme and proteinase K for one hour at 37^{o}C in order to lyse host cells and release the prophage if present. Next, a spin column based phage DNA extraction kit (Norgen Biotek Corp.

Ontario, Canada) was used to extract DNA from the treated pellet and the supernatant according to manufacturer's instructions.

Real-Time PCR Amplification:

The purified phage DNA was then amplified using real-time PCR. Reagents for PCR are listed in Table 2. The below primer set was previously used to detect GIL01, a gene that has been found in prophages within *Bacillus thuringiensis* [14]. PCR parameters included 40 cycles of denaturation at 94^{o}C, annealing at 55^{o}C, extension at 72^{o}C, and a melt curve analysis was generated at the end of every extension cycle.

Table 2: Reagents and primer sets used for PCR amplification.

Reagents:	SYBR Green (Sigma-Aldrich, St. Louis, MO) 2X Sensimix (Bioline, Taunton MA) 1.75 mM $MgCl_2$ 0.5 µg DNA template
Primers:	5′-GGATCCATGTTGACGCCAAGGG 5′-AAGCTTCAGTCATCCTTCTTCCC

DNA Sequencing:

DNA was sent along with the above primers to the genome center at UC Davis for sequencing, however it was found that we were unable to collect enough quality phage DNA for sequencing.

DISCUSSION:

Due to technical difficulties, and limited time and resources, we were unable to isolate quality phage DNA for sequencing, nor were we able to characterize the phage morphologically through electron microscopy in the two year timespan. The project was inherited by an incoming student, who has since been working on sequencing, and host range and phage sensitivity (outlined in future directions) , as well as sequencing of the genome.

FUTURE DIRECTIONS:

Study 1: Testing the ability of our novel bacteriophage to lyse *Bacillus cereus* cells.

Rationale: We hypothesize that the phage we discovered could be used in a cocktail as a way to safely reduce the number of pathogenic *Bacillus cereus* in dairy products. A cocktail is a mixture of several different phages introduced into an environment [15]. Although the use of phages in the food industry is being explored, there have been few studies done studying the effectiveness of phage cocktails on *Bacillus cereus* in dairy products. Furthermore, since there is such variety among families of phages, it is important to test the lytic properties for each.

To make a good candidate for cocktails in this study, the phage must quickly go into the lytic phase, and not become lysogenic for long periods of time. It is important to test the time it takes a phage to lyse the host cell because if phages are added to cocktails that do not lyse quickly enough, they essentially are dead weight. This study could be used to determine the time it takes the phage discovered in our previous study to lyse a *Bacillus cereus* cells in a liquid culture. In order to be considered effective for

our purposes: the phage must be able to lyse all bacteria in a given sample within three hours.

STUDY 1 DESIGN OVERVIEW: In order to test the lytic properties of our bacteriophage, we would grow *B. cereus* ATTC 14579 in a liquid culture as previously described, and inoculate the broth culture with 10^9 P.F.U [16], and record optical density, taking recordings every 20 minutes for 2 hours: after 2 hours take OD readings every 5 minutes for 30 minutes. As soon as a decrease in optical density is observed we would begin taking readings every 5 minutes until the optical density is consistent for three consecutive readings.

POSITIVE EXPECTED OUTCOME: We would expect the optical density to begin to decrease once the phage has had time to go through the lytic cycle (Figure 1). The trend should be that as the phages infect the bacterial cells, the OD will remain constant. Once the cells begin to go through lytic phase, the OD will begin to steadily drop. As the phages replicate within the cells, and they become more numerous the OD will drop at a quicker rate as they lyse more cells. Eventually all of the cells will have been lysed, and the OD will remain constant.

We expect to see the same trend in the lysozyme positive control. In the negative control we expect to see an increase in OD as the bacterial cells continue to divide.

CONTINGENCY PLAN: It is possible that our hypothesis will prove to be null, the phage that previously collected will not be able lyse *B. cereus* within three hours. If this occurs, we would deal with it in two ways. The first, is to go back to environmental sampling. As previously stated, phages are quite numerous, and therefore it is still possible that some

phages that have not yet been identified will meet our criteria. The second method would be to look back through the literature at cocktails that have been used in the past with phages that have not been characterized, test the phages individually, and combine the most effective ones in a single cocktail.

Study 2: Determination of host range of a novel phage found to infect *Bacillus cereus*

RATIONALE: In addition to being able to quickly lyse *B. cereus,* we concluded that an optimal candidate for a phage cocktail would also need to have a very narrow range of hosts to ensure that only toxigenic strains of *Bacillus* are being targeted. Most phages only infect one species of bacteria [5]. However we want to ensure that none of the phages are able to infect hosts of the same genus but different species.

STUDY 2 DESIGN OVERVIEW: In order to determine the host range, we would use the agar overlay method described above. Instead of *Bacillus cereus*, we would plate other members of the *Bacillus* genus that are closely related to *B.cereus.* Plaques will only form if the phage can infect this species of *Bacillus*. Since we previously discovered this phage using *Bacillus cereus* ATCC14579*,* an appropriate positive control would be to plate the phages on a lawn of *Bacillus cereus* ATCC14579. As a negative control, we will plate *Bacillus cereus* ATCC 14579 with no phages. As test organisms, *Bacillus subtillis*, *Bacillus thuringiensis subspecies israelnsis* and *Bacillus thuringiensis var kurstaki* could be used*.* These were chosen for their genetic and phenotypic relatedness to *Bacillus cereus* but they are not known human pathogens [2].

POSITIVE EXPECTED OUTCOME: If the results go according to plan, there should be no plaques on any of the plates except for the plate containing *Bacillus cereus*. The

plaques, as mentioned, can vary in size, but the top layer of agar should appear turbid, and a plaque will appear to be a small circular clearing. It is possible, however that the phage could infect multiple hosts. If this were the case, the phage would not be excluded from being a possibility for a cocktail, but it would mean that is less desirable from one that does not infect any of these bacteria besides *Bacillus cereus*.

CONTINGENCY PLAN: As mentioned in study 1, it is possible that the phage we have identified will not fit our parameter for host range. In this instance, we would again go back to environmental sampling. We believe that is the preferred method to find new cocktail prospects, because it also allows us to discover uncharacterized phages that are possibly more effective at lysis of *B. cereus.* If that proves to be unsuccessful, we could again comb through the literature and find phages that have been successfully used in cocktails, test them, and combine the phages that meet our criteria.

References:

1. Bottone, E.J., *Bacillus cereus, a volatile human pathogen.* Clin Microbiol Rev, 2010. **23**(2): p. 382-98.
2. Helgason, E., et al., *Bacillus anthracis, Bacillus cereus, and Bacillus thuringiensis--one species on the basis of genetic evidence.* Appl Environ Microbiol, 2000. **66**(6): p. 2627-30.
3. Ibrahim, M.A., et al., *Bacillus thuringiensis: a genomics and proteomics perspective.* Bioeng Bugs, 2010. **1**(1): p. 31-50.
4. Endersen, L., et al., *Phage Therapy in the Food Industry.* Annual Review of Food Science and Technology, Vol 5, 2014. **5**: p. 327-349.
5. Lee, W.J., et al., *Isolation and characterization of phages infecting Bacillus cereus.* Lett Appl Microbiol, 2011. **52**(5): p. 456-64.
6. Ackermann, H.W., *Frequency of morphological phage descriptions in the year 2000. Brief review.* Arch Virol, 2001. **146**(5): p. 843-57.
7. Gottlieb, P., et al., *In vitro replication, packaging, and transcription of the segmented double-stranded RNA genome of bacteriophage phi 6: studies with procapsids assembled from plasmid-encoded proteins.* J Bacteriol, 1990. **172**(10): p. 5774-82.
8. Sulakvelidze, A., Z. Alavidze, and J.G. Morris, Jr., *Bacteriophage therapy.* Antimicrob Agents Chemother, 2001. **45**(3): p. 649-59.
9. Brussow, H., *Phage therapy: the Escherichia coli experience.* Microbiology, 2005. **151**(Pt 7): p. 2133-40.
10. Chan, B.K., S.T. Abedon, and C. Loc-Carrillo, *Phage cocktails and the future of phage therapy.* Future Microbiol, 2013. **8**(6): p. 769-83.
11. O'Flynn, G., et al., *Evaluation of a cocktail of three bacteriophages for biocontrol of Escherichia coli O157:H7.* Appl Environ Microbiol, 2004. **70**(6): p. 3417-24.
12. Wall, S.K., et al., *Phage therapy to reduce preprocessing Salmonella infections in market-weight swine.* Appl Environ Microbiol, 2010. **76**(1): p. 48-53.
13. Edgar, R., et al., *High-sensitivity bacterial detection using biotin-tagged phage and quantum-dot nanocomplexes.* Proc Natl Acad Sci U S A, 2006. **103**(13): p. 4841-5.
14. Verheust, C., N. Fornelos, and J. Mahillon, *The Bacillus thuringiensis phage GIL01 encodes two enzymes with peptidoglycan hydrolase activity.* Fems Microbiology Letters, 2004. **237**(2): p. 289-295.
15. Zhang, J., et al., *Development of an anti-Salmonella phage cocktail with increased host range.* Foodborne Pathog Dis, 2010. **7**(11): p. 1415-9.
16. Stanisich, V.A., *The properties and host range of male-specific bacteriophages of Pseudomonas aeruginosa.* J Gen Microbiol, 1974. **84**(2): p. 332-42.